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(54) Title: REGULATION OF CANCER-CAUSING TYROSINE KINASES BY POTASSIUM ION CONDUCTANCE

(57) Abstract

The present invention provides a method for treating protein tyrosine kinase-mediated conditions by administering to a mammal an agent that increases cell membrane potassium ion conductance. Protein tyrosine kinase-mediated conditions include psoriasis, atherosclerosis and cancers. In one embodiment the agent is a compound that activates endogenous potassium ion channels. In a second embodiment, the agent is a compound which reacts with potassium ion to form a membrane-permeant complex. In a preferred embodiment, the agent is a potassium ion channel protein administered via incorporation of a DNA molecule encoding the protein into the cellular genome. The present invention also includes a pharmaceutical composition comprising a vector containing a gene encoding a potassium ion channel protein in a form suitable for administration to a mammal. In yet another embodiment, the present invention includes a method or assay for determining the activity of compounds which are potential potassium ion channel activators or inactivators. The present invention also comprises a method for determining the virulence of cancerous cells by measuring the potassium ion conductance of the cells.

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REGULATION OF CANCER-CAUSING TYROSINE KINASES BY POTASSIUM ION CONDUCTANCE

BACKGROUND OF THE INVENTION

Protein tyrosine kinases (PTKs) are enzymes which catalyze the phosphorylation of protein tyrosine residues. Protein tyrosine phosphorylation plays a primary role in the transduction of external biochemical signals to specific sites in the cell interior (Pelech S.L., *Curr. Biol.* 3:513-515 (1993)). An important class of PTKs includes transmembrane receptor proteins with an extracellular ligand binding domain and an intracellular PTK active site. Signal transduction is initiated by binding of a specific ligand to the receptor at the exterior surface of the cell, which then activates the PTK domain toward intracellular substrates (Schlessinger, J. *et al.*, *Neuron* 9:383-91 (1992)). A second class of PTKs includes cytosolic enzymes which are downstream carriers of the signal initially received at the cell membrane (Bolen, J.B, *Oncogene* 8:2025-2031 (1993)). A role for PTKs has been established in processes including cell growth, differentiation and division (Cadena *et al.*, *FASEB J.* 6:2332-2337 (1992)). Maintaining cellular homeostasis thus requires tight regulation of PTK activity, and cells employ a variety of regulatory mechanisms (Rodrigues *et al.*, *Curr. Opinion Gen. Dev.* 4:15-24 (1994)). Circumvention of these regulatory mechanisms is implicated in the development of a wide range of diseases, including cancers, psoriasis, atherosclerosis and autoimmune disorders. Thus the inhibition of PTK activity or subsequent steps in the signaling processes initiated by PTKs is an attractive goal in the development of treatments for proliferative diseases.

REGULATION OF CANCER-CAUSING TYROSINE KINASES BY POTASSIUM ION CONDUCTANCE

BACKGROUND OF THE INVENTION

Protein tyrosine kinases (PTKs) are enzymes which catalyze the phosphorylation of protein tyrosine residues. Protein tyrosine phosphorylation plays a primary role in the transduction of external biochemical signals to specific sites in the cell interior (Pelech S.L., *Curr. Biol.* 3:513-515 (1993)). An important class of PTKs includes transmembrane receptor proteins with an extracellular ligand binding domain and an intracellular PTK active site. Signal transduction is initiated by binding of a specific ligand to the receptor at the exterior surface of the cell, which then activates the PTK domain toward intracellular substrates (Schlessinger, J. *et al.*, *Neuron* 9:383-91 (1992)). A second class of PTKs includes cytosolic enzymes which are downstream carriers of the signal initially received at the cell membrane (Bolen, J.B, *Oncogene* 8:2025-2031 (1993)). A role for PTKs has been established in processes including cell growth, differentiation and division (Cadena *et al.*, *FASEB J.* 6:2332-2337 (1992)). Maintaining cellular homeostasis thus requires tight regulation of PTK activity, and cells employ a variety of regulatory mechanisms (Rodrigues *et al.*, *Curr. Opinion Gen. Dev.* 4:15-24 (1994)). Circumvention of these regulatory mechanisms is implicated in the development of a wide range of diseases, including cancers, psoriasis, atherosclerosis and autoimmune disorders. Thus the inhibition of PTK activity or subsequent steps in the signaling processes initiated by PTKs is an attractive goal in the development of treatments for proliferative diseases.

In particular, it is established that many cancers result from the expression of oncogene-encoded proteins (oncoproteins) following incorporation of retrovirus-derived DNA into the genome of the host cell. Oncogenes encoding PTKs constitute the largest single class of identified oncogenes and are known or suspected causative agents in many cancers, including colon cancers, brain cancers, lymphomas, breast cancer, ovarian cancer and leukemias. Although similar in most respects to normal cellular proteins, these oncoproteins may lack a regulatory amino acid sequence which in normal cellular proteins downregulates activity. In other cases as simple a modification as a single point mutation can thwart the regulatory mechanism. Often the oncoprotein is structurally indistinguishable from the native protein but is expressed at levels which overwhelm the cellular regulatory machinery. As PTKs are frequently involved in signaling processes switching on cell growth, the unregulated PTK activity of the oncoproteins can lead to the uncontrolled cell growth characteristic of cancer. Thus, inhibiting protein tyrosine kinase activity in cancer cells is believed to slow or halt their growth and proliferation.

PTK activity has also been implicated in the process of metastasis, by which cancer cells spread from the originally affected tissue to other parts of the body (Scholar *et al.*, *Cancer Letters* 87:159-162 (1994)). Tumor metastasis is a leading cause of cancer morbidity and mortality. Among the essential steps in metastasis is tumor invasion of the basement membrane, requiring adhesion of cells to this membrane, enzymatic digestion of a portion of the membrane, and passage of the cells through the resulting hole in the basement membrane. This invasion process depends upon poorly characterized signaling pathways that include PTKs, and the potent PTK inhibitor

genistein was shown to inhibit basement membrane invasion in a murine mammary carcinoma cell line. There is a need for additional inhibitors of PTK activity for the inhibition of tumor metastasis.

The role of protein tyrosine kinase activity in disease development has prompted considerable effort toward the design and synthesis of small molecule inhibitors of PTK activity (Levitzki *et al.*, *Science 267*:1782-1788 (1995)). Many of the synthetic PTK inhibitors are derived from natural products with broad PTK inhibitory activity, including the highly toxic genistein, and have demonstrated promise as therapeutic agents. For example, certain potent synthetic epidermal growth factor receptor (EGFr) inhibitors have proved capable of suppressing cell growth when applied to cultured cancer cells overexpressing EGFr (Buchdunger *et al.*, *PNAS 91*:2334 (1994)). Other compounds are able to reverse transformation by v-src in cultured chicken lens cells (Agbotounou *et al.*, *Mol. Pharmacol. 45*:922 (1994)). Compounds which inhibit platelet-derived growth factor receptor (PDGFr) are known to inhibit the proliferation of rabbit vascular smooth muscle cells and may be useful in the treatment of atherosclerosis (Bilder *et al.*, *Am. J. Physiol. 260*:C721 (1991)).

An obstacle facing the development of small molecule PTK inhibitors as therapeutic agents, however, is lack of selectivity. The importance of PTK activity in such a variety of signaling processes requires that PTK inhibitors used as drugs be narrowly targeted to the PTK of interest, so that only cells with high levels of the target PTK will be significantly affected. Tissue selectivity is also highly desirable to minimize adverse effects in healthy tissues. For example, the insulin receptor is a PTK, and its inhibition in otherwise healthy cells may lead to diabetes. Many chemotherapeutic agents for cancer treatment are of limited use due to the severe toxicity of

these drugs toward healthy tissue, and a primary focus in the development of novel cancer treatments is localization of activity to the disease site. Thus a major thrust of ongoing work in the area of small molecule PTK inhibitors is improvement of selectivity (Levitzki, *et al.* (1995), *supra*).

Much current activity focuses on treatment of disease via a protein expressed selectively in the affected cells by incorporating a DNA sequence encoding the protein into the cellular genome. The first examples of this approach were directed toward diseases arising from a genetic enzyme deficiency and included efforts to treat severe combined immunodeficiency by introducing the gene for adenosine deaminase into bone marrow (Cornetta *et al.*, *Prog. Nuc. Acid Res.* *36*:311-322 (1989)). Another strategy is "prodrug" gene therapy, in which the encoded protein is an enzyme which converts a nontoxic drug precursor into the active drug, thus providing a high concentration of the active drug at the disease site but considerably lower levels elsewhere. An example is administration of the herpes simplex thymidine kinase gene in conjunction with the drug ganciclovir, a treatment directed toward inhibition of DNA synthesis in cancer cells (Chen *et al.*, *PNAS* *91*:3054-3057 (1994); Smythe *et al.*, Thirty-first Annual Meeting of the Society of Thoracic Surgeons 52: A (1995)). A third approach involves the expression of a noncatalytic protein which influences some aspect of cellular activity, either alone or in tandem with drug therapy. An example is the selective targeting of erythrocytes to tumor cells by expression on the exterior of the erythrocytes a surface recognition protein selective for the tumor cells (U.S. Patent 5,399,346). None of these approaches, however, has yet given rise to a therapeutic method in routine clinical use. Thus, the need to augment the arsenal of anticancer therapies remains.

SUMMARY OF THE INVENTION

The present invention overcomes the problems associated with the lack of selectivity of small molecule PTK inhibitors. It is based on the discovery that the regulation of cell membrane potassium ion conductance regulates PTK activity and can be an effective mechanism for controlled and even selective PTK inhibition. Thus, the present invention provides a method for treating PTK-mediated diseases by administering to a mammal an agent which increases cell membrane potassium ion conductance. The method of the present invention inhibits the progression of disease via the inhibition of cellular PTK activity by increasing cell membrane permeability to potassium ions.

In one embodiment of the present invention, the disease is a cancer, such as those caused by expression of a PTK-encoding oncogene. In a particular embodiment, cell membrane potassium ion conductance is increased by introducing at the cell membrane a protein functioning as a potassium ion channel. In this embodiment, the method includes contacting the cancer cells with a vector containing a polynucleotide molecule encoding a potassium ion channel protein. The vector is capable of transfecting the cancer cells with the polynucleotide molecule which results in expression of the potassium channel protein. In another embodiment, a drug which increases the activity of native cellular potassium channels is delivered to the diseased cells. In another embodiment, the drug delivered to the diseased cells increases cell membrane permeability to potassium ions by providing a means of conductance other than passage through a potassium ion channel protein, for example, via a complex with a potassium-selective ionophore. A particular advantage of the present invention is the ability to selectively target the therapeutic agent to the diseased cells.

The present invention also includes a pharmaceutical composition comprising a vector containing a gene encoding a potassium ion channel protein in a form suitable for administration to a mammal.

In yet another embodiment, the present invention includes a method or assay for determining the activity of compounds which are potential potassium ion channel activators or inactivators. The method comprises: (1) expressing a potassium ion channel protein in a cell, (2) contacting the cells with an agent which increases cellular levels of protein tyrosine phosphorylation, (3) contacting the cells with the compound of interest, and, optionally, (4) comparing protein tyrosine phosphorylation levels of these cells with those of control cells treated according to steps (1) and (2) only (e.g., not contacted with the compound of interest). The level of cellular protein tyrosine phosphorylation is generally inversely correlated with the activity of the potassium ion channels. Thus, cellular phosphotyrosine levels will be decreased by a potassium ion channel activator and increased by a potassium ion channel inactivator.

The present invention also comprises a method for determining the virulence of cancer cells by measuring the potassium ion conductance of the cells. The potassium ion conductance of a transformed cell relative to a cell from the same tissue which does not display the transformed phenotype provides a measure of the PTK activity of the transformed cell, which is a measure of tumor malignancy and virulence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph comparing protein phosphotyrosine levels in two samples of human embryonic kidney cells, one expressing v-src alone, and the other co-expressing v-src and Kv1.3. In this and subsequent Figures, the indicated

density units are a direct measure of the amount of the indicated protein, and the result from one set of cells is scaled to 100 density units.

Figure 2 is a graph comparing protein tyrosine phosphorylation levels in cells expressing EGFr alone, cells expressing Kv1.3 alone and cells co-expressing Kv1.3 and EGFr.

Figure 3 is a plot of the time course of total cellular protein tyrosine phosphorylation in pervanadate-treated cells not expressing Kv1.3 and in pervanadate-treated cells expressing Kv1.3.

Figure 4 is a graph demonstrating that co-expression of Kv1.3 and v-src has no effect on the level of expression of v-src protein compared to cells expressing v-src alone.

Figure 5 compares total cellular protein content for control cells, cells expressing v-src only and cells co-expressing v-src and Kv1.3.

Figure 6 is a graph comparing cellular protein tyrosine phosphorylation levels in control cells, cells expressing v-src alone, cells expressing the nonconducting Kv1.3 mutant W386F Kv1.3 alone, and cells co-expressing v-src and W386F Kv1.3.

Figure 7 compares phosphotyrosine levels in three sets of pervanadate-treated cells: control cells expressing neither Kv1.3 or Y449F Kv1.3, cells expressing Kv1.3 and cells expressing Y449F Kv1.3.

Figure 8 is a graph showing the effect of added valinomycin on the phosphotyrosine level of pervanadate-treated cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for inhibiting the development of PTK-mediated diseases by administration of an agent which increases cell membrane potassium ion conductance, thus inhibiting PTK activity.

The agent may be a small molecule drug, such as a potassium ion transport antibiotic or a compound which increases the activity of potassium ion channel proteins, or a potassium ion channel protein administered, for example, via cell transformation, transfection, or infection with a vector containing a polynucleotide molecule encoding the potassium ion channel. The invention further provides a method for determining the activity of potential drugs which interact with potassium channels. Moreover, the invention provides a method for determining the virulence of cancer cells by measuring the potassium ion conductivity of the cells.

In the context of the present invention "proliferation" of a tumor, cancer cells or noncancerous cells is intended to include increase in cell or tumor size and/or number of cells. "Inhibition" of proliferation of cancer cells or tumor is therefore intended to include inhibition of an increase in cell or tumor size or inhibition of an increase in cell number and includes a decrease in the rate of proliferation, as well as a decrease in cell or tumor size or cell number. "Cancer cells" are intended to encompass any and all cancer cells characterized by uncontrolled growth and lack of contact inhibition and is intended to encompass nontumorous as well as tumorous cells, including tumorous cells which expand locally by invasion or systemically by metastasis. "Contacting" is intended to include methods of bringing the agent which increases cell membrane potassium ion conductance, such as a virus comprising a potassium ion channel gene, into immediate contact with the target cells so that the virus or vector, for example, is internalized by the cells and the potassium ion channel gene is expressed therein. The term "transfecting" means the introduction of an appropriate vector into a eukaryotic cell with which it is brought into contact. In the method of the present invention, a sufficient number of cells are

transfected resulting in a significant inhibition of cell proliferation. Accordingly, the amount of virus or vector administered should take into consideration the route of administration and should be such that the virus or vector will transfect a sufficient number of cells so contacted as to achieve the desired inhibition.

"Polynucleotide molecule encoding a potassium ion channel protein" is intended to mean a DNA or RNA sequence which when taken up by the host cell results in transcription, translation and expression of an active potassium ion channel protein. When the polynucleotide molecule is a DNA sequence, this can be incorporated directly into the cellular genome (which includes episomal expression). When the polynucleotide molecule is an RNA sequence, intracellular transcription of the sequence into DNA precedes incorporation into the cellular genome. The term is intended to include potassium ion channel genes or sequences, including greater and lesser sequences and mutations thereof (such as allelic variants), whether isolated from natural sources or synthesized in whole or in part, as long as the gene or sequence is capable of being expressed into a protein having the characteristic function of a potassium ion channel protein, i.e., the transport of potassium ions through the cell membrane. For the purposes of the present invention, the potassium ion channel protein gene can be from any suitable source, e.g., mammalian brain tissue. In a particularly preferred embodiment, the polynucleotide molecule is isolated from human tissue, thereby minimizing the risk that the expressed protein will elicit an immune response in the patient.

Likewise, "potassium ion channel protein", where it is caused to be expressed through recombinant DNA technology, is defined herein to include endogenous or wild-type proteins isolated from an animal such as a human. However, other mammalian proteins could be used as well, including

mouse, rat, guinea pig or primate. In view of the highly conserved nature of these proteins across widely varying animal species, proteins from other animals could be used as well, including, for example, *Drosophila*.

The invention is based on the discovery that co-expression of an oncogene encoding a protein tyrosine kinase and a gene encoding a potassium channel in cultured cells resulted in the suppression of PTK activity within the cells. As it is well established that unregulated PTK activity leads in many cases to the uncontrolled growth characteristic of transformed cells, inhibition of PTK activity in cancer cells can have the effect of halting or slowing the growth of cancer cells as well as preventing tumor metastasis. As described herein, experiments demonstrate suppression of PTK activity in cells co-expressing the PTK-encoding oncogene v-src and voltage-regulated potassium channel Kv1.3. Furthermore, expression of Kv1.3 in cells, without co-expression of v-src, resulted in suppression of endogenous PTK activity. Kv1.3 has also been shown to suppress protein tyrosine phosphorylation when co-expressed with an oncogene-encoded epidermal growth factor receptor (EGFr).

Studies also showed that v-src levels in cells co-expressing v-src and Kv1.3 were identical to those of cells expressing only v-src. Thus Kv1.3 does not inhibit v-src activity by suppressing expression of the oncoprotein. Total cellular protein content was examined in control cells, cells expressing v-src alone, cells expressing Kv1.3 alone and cells co-expressing v-src and Kv1.3. No differences in total cellular protein were discernible among these four groups of cells. Thus the expression of Kv1.3 does not reduce levels of cytosolic proteins which are substrates of v-src. This would also have the effect of reducing apparent v-src activity.

Additional experiments demonstrated that the inhibition of v-src activity by Kv1.3 requires that the potassium channel is localized at the cell membrane and that it is conductive. Thus co-expression of v-src and a nonconductive Kv1.3 mutant resulted in no diminution of protein tyrosine phosphorylation compared to cells expressing v-src alone. Co-expression of v-src and Kv1.3 in cells treated with the Golgi complex inhibitor brefeldin-A (Klausner *et al.*, *J. Cell Biol.* 116:1071-1080 (1992)) also resulted in no measurable decrease in protein tyrosine phosphorylation relative to brefeldin-A treated cells expressing v-src alone. Because a primary function of the Golgi complex is targeting of newly synthesized proteins to specific sites within the cell, in this experiment expressed Kv1.3 was not directed to the cell membrane. Taken together, these results indicate that suppression of v-src activity requires an increase in cell membrane potassium ion conductance, as is provided by addition of a functional potassium channel spanning the cell membrane.

Recent results also show that increasing cell membrane potassium ion conductance by a means independent of potassium ion channels can also inhibit PTK activity. Treatment of cells with valinomycin, a potassium-selective ionophore that forms a membrane-permeant complex with potassium ion, results in inhibition of endogenous PTK activity. This result demonstrates that a variety of methods for increasing cell membrane potassium ion conductance can inhibit PTK activity.

Kv1.3 is thus an effective PTK inhibitor in two different models of PTK-mediated cell over-proliferation implicated in cell transformation (Perkins *et al.* in Cancer: Principles and Practice of Oncology, DeVita *et al.*, eds., Lippincott Co., Philadelphia (1993)). Cells expressing v-src provide a model for expression of an

intracellular PTK lacking a regulatory domain, while those expressing EGFr provide a model for the overexpression of a growth factor receptor, as has been observed in 25 to 30 per cent of human primary breast cancers (Slamon *et al.*, *Science* 244:707-712 (1989)).

PTKs can generally be classified into three distinct groups: receptor PTKs, src-like PTKs and abl-like PTKs (Rodrigues *et al.* (1994), *supra*). Of these, the src-like and abl-like PTKs are most similar and share several key features, including the structural arrangement of certain domains. Thus Kv1.3 has been shown to effectively inhibit PTKs from two of these three subclasses, and the two subclasses represented display the greatest divergence in properties. This suggests that Kv1.3 is capable of inhibiting a broad spectrum of PTKs.

One embodiment of the present invention relates to the treatment of a PTK-mediated condition in which the affected cells have a sufficient density of endogenous potassium ion channels that cell membrane potassium ion conductance may be increased in a physiologically significant amount by the administration of drugs which activate potassium ion channels. Brain cancers and lymphomas are examples of preferred targets for such a strategy. Compounds which are suitable for use as potassium channel openers include the family of substituted benzimidazolone derivatives as described in European Patent Application No. 0477819A2 (1992), which is incorporated herein by reference. In one embodiment of the present invention the benzimidazolone derivative 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2*H*-benzimidazole-2-one is administered to the cancerous cells. This compound has been shown to open calcium-activated large conductance potassium channels from rat brain (McKay, M.C., *et al.*, *J. Neurophysiology* 71:1873-1881, (1994)). Another suitable drug is the potassium channel opener minoxidil, which is a widely used

antihypertensive drug (Meisheri *et al.*, *J. Pharmacol. Exp. Ther.* 245:751-760 (1988)). Other known potassium ion channel activators of use in this method include cromakalim and lemakalim (Carl *et al.*, *Pfluegers Arch.* 421:67-76 (1992)).

Another embodiment of the present invention is a method which comprises contacting the cells, such as cancer cells, with an agent which increases cell membrane potassium ion conductivity by means other than increasing activity of endogenous potassium channels. In one embodiment, the agent is a potassium ion complexing agent, which upon interaction with a potassium ion yields a membrane-permeant complex. This complex can then effect potassium ion transport into or out of the cell. Complexing agents which are specific or highly selective for potassium ions are suitable for use in this method and include polypeptide ionophores such as valinomycin and enniatin B (Dobler, Ionophores and Their Structures, Wiley-Interscience, New York (1981)), naturally occurring cyclic polyethers such as monactin, nonactin, dinactin, trinactin and tetranactin (Dobler (1981), *supra*), and such synthetic polyethers as 18-crown-6 and its derivatives and cryptate-222 (Cotton *et al.*, Advanced Inorganic Chemistry, Fifth Ed., Wiley Interscience, New York, 135-136 (1988))

The preferred embodiment of the present invention is a treatment of a PTK-mediated condition in a mammal in which the agent increasing cell membrane potassium ion conductance is a potassium ion channel protein. The method of administration of this agent includes contacting a cell, such as a cancer cell, with a vector containing a polynucleotide molecule encoding the potassium channel to achieve transfection, transformation or infection of the cell and incorporation of the potassium channel-encoding DNA sequence into the cellular genome. This results in expression of functional, membrane-localized potassium ion

channel proteins, thereby increasing the cell membrane potassium ion conductance. Potassium ion channel proteins suitable for use in this method include the family of voltage-dependent potassium ion channel proteins. This family of potassium ion channel proteins includes the *Shaker*, *Shab*, *Shaw*, and *Shal* subfamilies of potassium ion channels from *Drosophila*, and their vertebrate homologues. These vertebrate homologues comprise the characterized potassium ion channel proteins Kv1.1-Kv1.7, Kv2.1, Kv3.1, Kv3.2, Kv3.3, Kv3.4, Kv4.1 and Kv4.2 (Chandy *et al.*, *Nature* 352:26 (1991)). Members of this group which have been cloned from rat brain include Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5 and Kv1.6 (Swanson, *et al.*, *Neuron* 4:929-939 (1990); Stuhmer *et al.*, *EMBO J.* 8:3235-3244 (1989)). Kv1.3 has been cloned from both human (Attali *et al.*, *FEBS Letters* 303:229-232 (1992); Douglass *et al.*, *J. Immunology* 144: 4841-4850 (1990)) and murine T-lymphocytes (Chandy *et al.*, *Science* 247:973-975 (1990); Grissmer *et al.*, *PNAS* 87:9411-9415 (1990)). The *Shaker* channel itself has also been cloned (Butler *et al.*, *Science* 243:943-947 (1989)). The contents of these articles are incorporated herein by reference.

In another embodiment of the present invention, the potassium ion channel protein administered to the animal is a modified version of a wild-type potassium ion channel protein produced by deletion, addition or substitution of amino acids of the wild-type channel. This embodiment encompasses alterations to wild-type potassium ion channels which increase and/or permit greater control of their activity under the conditions of the therapeutic application. Inactivation of Kv1.3 has been shown to occur in a time-dependent fashion in the presence of v-src and other PTKs, indicating that Kv1.3 is inactivated by phosphorylation of a constituent tyrosine residue. Thus, a particularly preferred embodiment of the method for

treatment of PTK-mediated conditions comprises administering to an animal a mutant potassium ion channel protein that is not inactivated by tyrosine phosphorylation, such as substituting one or more tyrosine residues with another amino acid, such as phenylalanine or met-tyrosine, or deleting the amino acid. Recent results demonstrate that substitution of the tyrosine at position 449 of Kv1.3 with phenylalanine yields a potassium ion channel protein which does not undergo pervanadate-induced inactivation. This implicates phosphorylation of tyrosine 449 in pervanadate-induced inactivation of wild-type Kv1.3. Thus, the mutant Y449F Kv1.3 is expected to be more active than wild-type Kv1.3 in cells exhibiting significant amounts of PTK activity. Mutants of Kv1.3 and its homologues in which multiple tyrosine phosphorylation sites have been removed, such as YYY111,112,113FFF Kv1.3, can also be used in the present method.

Other studies of potassium ion channel protein inactivation suggest additional means of increasing channel activity. Kv1.2, for example, has also been shown to be inactivated by tyrosine phosphorylation, and a Kv1.2 mutant resulting from substitution of a phenylalanine residue for the tyrosine residue at position 132 is significantly less susceptible to PTK-mediated inactivation (Huang *et al.*, *Cell* 75:1145-1156 (1993)). In addition, a Kv1.3 mutant substituting a tyrosine residue for the histidine residue at position 401 displayed a slower rate of C-inactivation compared to the wild-type protein (Kupper *et al.*, *Pfluegers Arch.* 430:1-11 (1995); Busch *et al.*, *Biochem. Biophys. Res. Comm.* 179:1384-1390 (1991)). Slower rates of C-type inactivation were also observed in several Kv1.3 mutants in which one or more serine or threonine residues were replaced with alanine (Kupper *et al.* (1995), *supra*). In another example, a potassium ion channel protein from rat kidney was inactivated via direct phosphorylation by

protein kinase C, which phosphorylates protein serine and threonine residues. Replacement of the serine residue at position 103 in this protein with alanine yielded a mutant which was unaffected by protein kinase C (Busch *et al.*, *Science* 255:1705-1707 (1992)). The foregoing examples of characterized mutant potassium ion channel proteins can be used in the present method. Given the extent of sequence homology among families of potassium ion channels, corresponding mutants of homologous channels can be made and used as well (Stuhmer *et al.* (1989), *supra*). The articles cited above are incorporated herein by reference.

Vectors suitable for transfection of the diseased cells with a polynucleotide molecule encoding a potassium ion channel are a variety of viruses rendered non-infectious, infectious for a single cycle, replication-deficient, or otherwise suitable for a gene therapy application, such as DNA viruses, including adenoviruses (Ginsburg (ed.), The Adenoviruses, Plenum Press, NY (1984)), herpes simplex virus type 1 (Shih *et al.*, *PNAS* *81*:5867 (1984)), cytomegalovirus (Mocarski *et al.*, in Viral Vectors, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 73-77 (1988)), and Epstein-Barr virus (Margolskee *et al.*, *Mol. Cell. Biol.* *8*:2837 (1988)) and retroviruses, including avian and murine sarcoma viruses (Ellis *et al.*, Gene Targeting with Retroviral Vectors, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1988)). Retroviral vectors have found the greatest use in clinical gene therapy protocols. The RNA-encoded product of these vectors can be expressed in cells actively synthesizing DNA, i.e. dividing cells, which can be an advantage in the selective targeting of cancerous cells, as such cells divide more rapidly than the noncancerous cells in the same tissue (Culver *et al.*, *Science* *256*:1550-1552 (1992)). The viral vector can be any such vector appropriate for introduction of a DNA sequence into eukaryotic cells, and

particularly mammalian, for example human, cells. The resulting vector must be capable upon integration into the cellular genome to cause transcription, translation and expression of active potassium ion channel protein in the host cell. In preferred embodiments of the present method, the viral genome can be altered in size, e.g. by insertion or deletion, and in sequence, e.g., by mutation, insertion or deletion, as desired or as necessary to render the viral vector suitable for therapeutic use, taking into account packaging restrictions as necessary, and to enable expression of the potassium ion channel gene in the transfected cell without causing systemic or significant viral disease in the patient.

Nonviral delivery of the potassium ion channel protein-encoding DNA molecule to cells with unregulated PTK activity in vivo is also encompassed by the present invention. For example, liposomes may be used to transport the polynucleotide across the cell membrane, followed by incorporation of the DNA sequence into the cellular genome. Immunoliposomes comprising an antibody specific for a surface protein expressed in the target cells can provide selective delivery of the DNA sequence to the disease site (Nassander *et al.*, *Cancer Res.* *52*:646-653 (1992)).

The ordinary skilled artisan is aware that different genetic signals and processing events control levels of nucleic acids and proteins/peptides in a cell, such as, for example, transcription, mRNA translation, and post-transcriptional processing. Transcription of DNA into RNA requires a functional promoter. The amount of transcription is regulated by the efficiency with which RNA polymerase can recognize, initiate, and terminate transcription at specific signals. These steps, as well as elongation of the nascent mRNA and other steps, are all subject to the effects of various other components also present in the cell, for example, by other proteins which

can be part of the transcription process or by concentrations of ribonucleotides present in the cell. Similarly, translation of mRNA requires, at the least, a ribosome binding site located at an appropriate distance from the start codon. Expression of a foreign nucleic acid sequence in a cell can be improved with post-translational modification of a resultant protein/peptide. Thus, production of a recombinant protein/peptide can be affected by the efficiency with which DNA is transcribed into mRNA, the efficiency with which mRNA is translated into protein, and the ability of the cell to carry out post-translational modification. Enhancer elements can also be incorporated into the vector to further increase the amount of protein expressed. The selection and incorporation into the vector of suitable elements required for efficient expression are, generally, considered routine and well within the ordinary skill in the art.

The present invention also encompasses contacting the vector comprising a polynucleotide molecule encoding a potassium ion channel protein with eukaryotic cells *in vitro* by using standard techniques including DNA viral or retroviral infection, lipofectamine infection, electroporation, calcium phosphate-mediated incorporation or microinjection (Old *et al.*, Principles of Gene Manipulation: An Introduction to Genetic Engineering, Blackwell Scientific Publications, Oxford (1989)). DNA viral or retroviral infection is a particularly preferred embodiment because of its high efficiency.

The PTK-mediated conditions to which the above described methods are applicable include a variety of diseases characterized by excessive cell proliferation. These include, but are not limited to, psoriasis, atherosclerosis, autoimmune disorders and cancers. These diseases can arise from unregulated PTK activity signaling cell growth and division. Cancers in which unregulated PTK

activity has been implicated include leukemias, lymphoma, glioma, brain cancer, colon cancer, breast cancer, ovarian cancer, thyroid cancer, skin cancers, stomach cancer, bladder cancer and prostate cancer (Wilks, *Adv. Cancer Res.* *60*:43-73 (1993); Perkins *et al.* in Cancer: Principles and Practice of Oncology, Fourth Edition, DeVita *et al.*, ed., J.B. Lippincott Co., Philadelphia (1993); Heruth *et al.*, *J. Cell. Biochem.* *58*:83-94 (1995); Fuller *et al.*, *Mutation Res.* *276*:299-306 (1992); Fox *et al.*, *Brit. J. Urology* *74*:214-220 (1994); Lofts *et al.*, *Cancer Treat. and Res* *61*:161-179 (1992)). The role of PTK activity in tumor metastasis indicates that agents inhibiting PTK activity by increasing cell membrane potassium ion conductance can also inhibit tumor metastasis (Scholar *et al.* (1993), *supra*).

Another embodiment of the present invention is a pharmaceutical composition appropriate for administration of a vector containing a polynucleotide molecule encoding a potassium ion channel protein to a host eukaryotic, preferably mammalian, cell *in vivo*. Such a composition comprises the vector itself and an appropriate carrier or diluent. The agent which increases cell membrane potassium ion conductance, including the vector, can be administered alone or in a pharmaceutically acceptable carrier and can take forms including but not limited to a tablet, capsule, aerosol, solution, ointment, injection or inhalant.

Means known in the art may be utilized to prevent or minimize release and absorption of the composition until it reaches the target cell, tissue, or organ, or to ensure time-release of the composition. In a preferred embodiment, the administration is a local administration at or near the site of cell proliferation. In pharmaceutical dosage forms, the compositions can be used alone or in combination with other pharmaceutically active compounds. For example, administration of the vector containing a polynucleotide sequence encoding a potassium ion channel

may be carried out in conjunction with the administration of an agent which increases the activity of the subsequently expressed potassium ion channel, such as minoxidil, cromakalim or lemakalim. Compositions suitable for *in vitro* administration to eukaryotic cells are also contemplated by the present invention to control cellular proliferation in culture.

The present invention additionally provides a method or assay for the *in vitro* evaluation of the activity of compounds which are potential potassium ion channel activators or inactivators. This method is based on the observed inhibition of PTK activity by increased cell membrane potassium ion conductance. Potassium ion channel activators would find use in the treatment of cell proliferative diseases as described above and serve as therapeutic neuroprotective agents. Rapid administration of potassium ion channel openers should be useful for attenuating excitotoxic damage that occurs following stroke and trauma. The methods currently used for screening and characterizing potassium ion channel openers are time-consuming and require highly trained physiologists. The present invention can considerably streamline the drug-screening process. Additional advantages of the present method include the ability to screen drugs that interact specifically with defined channel subtypes in heterologous expression systems as well as endogenously expressed channels.

The method for assaying potassium ion channel regulatory activity in cells by a compound comprises the steps of (1) contacting cells with an agent which increases potassium ion channel activity; (2) contacting the cells of step (1) with an agent which increases tyrosine phosphorylation, (3) contacting the cells of step (2) with the compound and (4) measuring tyrosine phosphorylation. In a preferred embodiment, the method is a modified enzyme-

linked immunosorbent assay (ELISA) comprising the steps of (1) transfecting cells in vitro for the expression of specific potassium ion channel proteins and PTKs, maintaining cells under conditions suitable for protein tyrosine phosphorylation to occur, (2) treating cells with a compound of interest, (3) incubating cells with reporter enzyme-coupled anti-phosphotyrosine antibodies or other labelled antibodies and (5) measuring phosphotyrosine levels. In one variant of this method the cells are not caused to express an exogenous PTK as indicated in step (1), but are instead treated with a protein tyrosine phosphatase inhibitor such as pervanadate or orthovanadate to enhance endogenous PTK activity. Comparison of phosphotyrosine levels of these cells with phosphotyrosine levels of control cells not treated with the compound of interest but otherwise identical provides a measure of the potassium ion channel activating ability of the tested compound. Phosphotyrosine levels in treated cells relative to the control are directly dependent upon the degree of activation or inactivation of the potassium ion channels.

The present invention also includes a method for the diagnosis of tumor virulence by determination of the potassium ion conductance of the cancer cells. Voltage-regulated potassium ion channels are inactivated by PTKs, while increasing the activity of potassium ion channels inhibits PTK activity. Thus, in the assessment of cancer cells from a given tissue, lower levels of potassium ion conductance correlate with higher levels of PTK activity while higher levels of potassium ion conductance correlate with lower levels of PTK activity. Tumors with enhanced EGFr expression, for example, are expected to have relatively low membrane potassium ion conductance relative to nontransformed cells from the same tissue. Such tumors are known to be highly malignant with low patient survival rates (Slamon, D.J., *et al.*, (1989), *supra*). An example of

the method for assessing the degree of cell transformation and malignancy comprises the steps of (1) removing cancerous cells from the patient, (2) preparing cell-attached membrane patches, and (3) measuring ion-induced currents across the cell membrane using standard electrophysiological techniques (Kupper *et al.* (1995), *supra*; Marom *et al.*, *Biophys. J.* 67:579-589 (1994)).

EXAMPLES

General Materials and Methods

The following procedures and materials were employed in Examples 1-9 below. Additional methods unique to a particular Example are provided with that Example.

cDNA expression vectors

All mammalian expression vectors for these experiments contained the cytomegalovirus promoter upstream from the coding region. The plasmid pRc-CMV (Invitrogen, San Diego, CA) was used as control vector for all experiments. Kv1.3 cDNA was prepared as previously described (Swanson, R. *et al.*, *Neuron* 4:929-939 (1990)), and was inserted into pRc-CMV (Bowlby, M.R. *et al.*, *J. Neurophysiol.* 73:2221-2229 (1995)). The cDNAs for v-src kinase (v-src) and the human epithelial growth factor receptor (EGFr) were prepared according to a published procedure (Qu, Z. *et al.*, *Neuron* 2:367-378 (1990)).

Cell culture and transfection procedures

Human embryonic kidney (HEK) cells were maintained in modified eagle medium (MEM) containing 2% penicillin/streptomycin and 10% fetal bovine serum (Gibco/BRL, Grand Island, NY). Cells were grown to confluency over one week, dissociated with trypsin-EDTA and

mechanical trituration, diluted in MEM to a concentration of approximately 600 cells/μl, and replated on Corning plastic dishes #25000. cDNAs coding for Kv1.3, v-src, EGFr or control vector were introduced into HEK 293 cells by lipofectamine transfection. Cells were transfected three to five days after recovery from cell passage, at 70-80% confluency. The total amount of cDNA used for transfection was the same for all control and experimental groups. Cells were transfected with 5 μg DNA/60 mm dish.

For Kv1.3/tyrosine kinase co-expression experiments, cells were transfected with a total of 10 μg DNA/60 mm dish, 5 μg DNA of each construct coding for Kv1.3 and tyrosine kinases. The potassium ion channel or tyrosine kinases alone groups were brought up to 10 μg DNA by the addition of 5 μg vector-control DNA. Co-transfection control cells were transfected with 10 μg vector noncoding DNA/60 mm dish. Cells were incubated for 5 hours with the lipofectamine/DNA mixture diluted in serum-reduced medium (OptiMEM, Gibco/BRL, Grand Island, NY). Transfection efficiency was monitored in parallel plates by staining for the β-galactosidase reaction product in Lac-Z expression plasmid transfected cells. Staining efficiency (blue cells) normally ranged from 70-90%. Expression of Kv1.3 could be detected immunochemically in as little as 24 hours and was sufficient to produce macroscopic currents in cell-attached membrane patches 24-72 hours after transfection.

Cell lysis and immunoprecipitation

Cells were harvested two days after transfection by lysis in ice-cold 1% Triton X-100 modified immunoprecipitation buffer containing protease and phosphatase inhibitors (25 mM Tris, pH 7.5; 150 mM NaCl; 100 mM NaF; 5 mM EDTA; 1 mM Na_3VO_4, 1% Triton X-100; 1 mM PMSF; 1 μg/mL leupeptin; 2 μg/mL aprotinin). The cell lysates were clarified by centrifugation (15,000 x g, 5

min., 4 °C). Immunoprecipitation of lysate proteins from the supernatant utilized a 2 hour incubation with antibody at 4 °C followed by an overnight incubation with Protein-A sepharose. The immunoprecipitates were washed 3 times with ice-cold 0.1% Triton X-100 modified immunoprecipitation buffer. Lysate samples and washed immunoprecipitates were diluted in SDS-gel loading buffer.

Western blot and autoradiogram procedures

Proteins were separated on 10% acrylamide gels by SDS-PAGE and electrotransferred to nitrocellulose blots. The blots were blocked in 5% nonfat milk and incubated overnight in primary antibody at 4 °C. The blots were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Corp., Arlington Heights, IL) for two hours at room temperature. Enhanced chemiluminescence exposure on XAR film was used to visualize labelled protein. The magnitude of the signal is directly related to the amount of HRP-conjugated secondary antibody. The film autoradiograms were analyzed by densitometry using a Biorad model GS-670 Imaging Densitometer. Relative densitometry values were shown to be linear by serial dilution of protein samples used for western immunoblotting.

Antibodies and reagents

Tyrosine-phosphorylated proteins were immuno-precipitated and detected by western blot with mouse monoclonal antibody 4G10 (Upstate Biochemical) The specificity of 4G10 was verified by preabsorption with phosphotyrosine. Tyrosine, phosphoserine and phosphothreonine did not affect anti-phosphotyrosine immunostaining. The antiphosphotyrosine antibody PY20 was used for western blot to detect tyrosine-phosphorylated proteins in the 4G10 immunoprecipitates. Immuno-

precipitated tyrosine phosphorylated proteins were further characterized by alkaline phosphatase treatment. Two specific rabbit polyclonal antisera raised against MaIE fusion proteins containing sequences specific to Kv1.3 were prepared as described (Cai, Y.-C. *et al.*, *J. Biol. Chem.* *268*:23720-23727 (1993)). Both antisera were used for western blotting and immunoprecipitation. EGFr expression was detected with a human-specific mouse monoclonal antibody. Src expression (v-src and c-src) was detected with a mouse monoclonal antibody. All other chemicals used for western blotting, immunoprecipitation and electrophysiology were purchased from Sigma Chemical Company (St.Louis, MO).

Example 1 Inhibition of the PTK activity of v-src by co-expression of KV1.3

Materials and Methods

Cell transfection with cDNA encoding v-src and Kv1.3 and western blot analysis of protein tyrosine phosphorylation were performed as described above.

Results

This example demonstrates that co-expression of v-src and the potassium ion channel protein Kv1.3 in cultured human embryonic kidney cells results in significant attenuation of PTK activity. Western blot analysis of cellular proteins phosphorylated on tyrosine was performed for four different cases: (1) control; (2) expression of v-src only; (3) expression of Kv1.3 only; (4) co-expression of v-src and kv1.3. The results are displayed in Figure 1. The control sample showed no detectable tyrosine-phosphorylated protein, indicating that endogenous tyrosine phosphorylation occurs at a very low level. Expression of Kv1.3 alone has no effect on the level of protein tyrosine

phosphorylation relative to the control. Expression of v-src alone results in a dramatic increase in protein tyrosine phosphorylation. The effect of v-src, however, is significantly and reproducibly attenuated when the oncogene is co-expressed with the Kv1.3 potassium ion channel protein. In two experiments, densitometry indicated that co-expression of Kv1.3 with v-src resulted in a 50% reduction in the level of protein tyrosine phosphorylation relative to the level observed when v-src was expressed alone.

Example 2 Inhibition of epidermal growth factor receptor by Kv1.3

Materials and methods

Transfection of cells with cDNA encoding EGFr and Kv1.3 and western blot analysis of protein tyrosine phosphorylation was performed as described above.

Results

This experiment demonstrates that co-expression of Kv1.3 with another oncogenic PTK, epidermal growth factor receptor (EGFr), also resulted in decreased protein tyrosine phosphorylation relative to expression of the oncogene alone. Western blot analysis of protein tyrosine phosphorylation was performed for two sets of cells, one expressing EGFr alone the other co-expressing EGFr and Kv1.3 (Figure 2). Relative to the EGFr-only sample, co-expression of Kv1.3 yielded a 56% reduction in protein tyrosine phosphorylation as measured by densitometry.

Example 3 Inhibition of endogenous PTK activity by Kv1.3 in the presence of pervanadate

Materials and Methods

Cells were transfected with cDNA encoding Kv1.3 and western blot analysis of protein tyrosine phosphorylation were performed as described above.

Pervanadate preparation and treatment

Pervanadate was prepared by a 2 minute incubation of 100 mM Na_3VO_4 and H_2O_2, followed by dilution to the appropriate concentration (0-250 μM, 0.0008% H_2O_2) in serum-free MEM. Cells were incubated with pervanadate in MEM for the times indicated. For electrophysiology experiments pervanadate (100 μM final) was prepared by diluting Na_3VO_4 in extracellular solution (see below) with H_2O_2 (0.003% final) for 20 minutes followed by the addition of 54 μM catalase for 5 minutes to quench residual H_2O_2. After achieving a cell-attached patch configuration, cells were treated with patch solution + 0.003% H_2O_2 + 54 μM catalase for 10 minutes to insure there was no effect of H_2O_2 alone. The bath was replaced with the pervanadate extracellular solution for the duration of the patch recording.

Electrophysiology experiments

For electrophysiology experiments, cells were grown as described above and transfected at 20-30% confluency with 1 μg DNA/ 35 mm dish. Patch clamp analysis was performed according to a published procedure (Kupper *et al.* (1995), *supra*).

Results

This experiment demonstrates that the presence of the potassium ion channel protein Kv1.3 inhibits the activity

of endogenous cellular PTKs. In normal cells, as observed in Example 1, protein tyrosine phosphorylation is minimal due to the activity of tyrosine phosphatases, enzymes which dephosphorylate phosphotyrosine residues. Pervanadate is a powerful, membrane-permeant inhibitor of tyrosine phosphatase. When tyrosine phosphatase activity is inhibited by pervanadate, tyrosine phosphorylation is an irreversible process and the level of protein tyrosine phosphorylation increases with time. The time course of protein tyrosine phosphorylation was followed by western blot analysis for two sets of cells, one set expressing Kv1.3 and a second lacking Kv1.3. Analysis was performed upon addition of pervanadate to the cells (time = 0 minute) and at times 5, 30 and 60 minutes subsequent to pervanadate addition, as indicated in Figure 3. While for both sets of cells the level of protein tyrosine phosphorylation increased with time, it is clear that levels were sharply reduced in the presence of Kv1.3. Thus the presence of Kv1.3 inhibits endogenous cellular PTKs as well as those encoded by oncogenes.

Electrophysiological studies directed at determination of potassium ion currents were conducted in the presence and absence of pervanadate and orthovanadate to determine any effect of these ions on potassium ion channel activity. The results of these studies revealed no change in the activity of expressed Kv1.3 in the presence of these ions.

Example 4 Effect of expression of Kv1.3 on the level of expression of v-src protein

Materials and methods

Cells were transfected with cDNA encoding Kv1.3 and v-src and grown as described above. Western blot analysis

and src-specific staining were performed as described above.

Results

This experiment demonstrates that co-expression of Kv1.3 has no effect on the level of expression of v-src protein. Western blot analysis was performed on four sets of cells: (1) control, (2) expression of v-src only, (3) expression of Kv1.3 only, (4) co-expression of v-src and Kv1.3 and the results are shown in Figure 4. Comparison of the control cells with those expressing v-src showed that an endogenous PTK in the control is very similar to v-src. When v-src is expressed, the level of this protein increases substantially. The presence of Kv1.3, however, has no effect on the level of this protein, as co-expression of v-src and Kv1.3 resulted in a level of v-src protein indistinguishable from the level found in the absence of Kv1.3. This experiment was designed to address whether the expression of KV1.3 causes a decrease in the level of co-expression of v-src protein, a possible mechanism for apparent v-src inhibition in the presence of Kv1.3. The results presented here clearly show that this is not the case.

Example 5 Effect of expression of Kv1.3 on total cellular protein content

Materials and methods

Cell transfection with cDNA encoding Kv1.3 and v-src and cell growth were performed as described above. Western blot analysis was as described. Total protein content was visualized by staining with Coomassie blue.

Results

This experiment showed that expression of v-src alone, Kv1.3 alone or co-expression of v-src and Kv1.3 had no effect on the total cellular protein distribution in comparison to a control in which neither protein is expressed, as shown in Figure 5. This experiment was designed to reveal whether expression of Kv1.3 interferes with the production of endogenous cellular proteins. A possible mechanism for the attenuation of v-src activity by Kv1.3 is the decrease in levels of the protein substrates for v-src. The present results indicate that this is not the case.

Example 6 Effect of a non-conducting Kv1.3 mutant on the activity of co-expressed v-src

Materials and methods

Transfection of cells with cDNA encoding the mutant W386F Kv1.3 and v-src, western blot analysis, and visualization of phosphotyrosine were performed as described above.

Preparation of W386F Kv1.3

Site-directed mutagenesis was employed in order to construct the mutant Kv1.3 channel W386F Kv1.3, in which tryptophan residue 386 was substituted with phenylalanine. A single polymerase chain reaction using a mutagenic primer and a wild-type primer was used to introduce the mutation. The PCR reactions were run in a thermocycler (Eri-Comp, Twin Block System, San Diego, CA) using Taq polymerase (Promega, Madison, WI). Each PCR product was cut sequentially with BsteII (Promega) and PstI (Boehringer Mannheim, Indianapolis, IN) with a phenol/chloroform extraction and ethanol precipitation between cuts. An identical protocol was used to cut wild-type Kv1.3 in pGEM

vector in order to construct a backbone. The desired pieces were purified (Gene-Clean II, Bio Labs 101, Vista, CA) from a 2% agarose gel. The mutagenic insert was ligated into the Kv1.3-pGEM backbone using T4 DNA ligase (Promega). The W386F Kv1.3 sequence between the BsteII and Pst1 sites was confirmed by use of an automatic sequencer. The entire sequence coding for the mutant channel was cut out of pGEM with HindIII and NcoI and ligated into the pRcCMV mammalian expression vector. The W386F Kv1.3 sequence between the BsteII and Pst1 sites in the pRcCMV vector was reconfirmed by use of an automated sequencer.

Results

This experiment demonstrates that Kv1.3 must be functional to inhibit the activity of v-src. The effects of Kv1.3 and the nonconducting Kv1.3 mutant W386F Kv1.3 on the activity of co-expressed v-src were directly compared by western blot analysis of protein tyrosine phosphorylation and are presented in Figure 6. As observed in Example 1, active Kv1.3 produced a significant decrease in the level of protein tyrosine phosphorylation in the presence of co-expressed v-src, while in the presence of the nonconducting mutant W386F Kv1.3 little or no attenuation of protein tyrosine phosphorylation was observed. Thus the presence of the Kv1.3 protein alone is not sufficient for PTK inhibition; the potassium ion channel protein must also be active.

Example 7 Effect of cytosol-confined Kv1.3 on the activity of co-expressed v-src

Materials and methods

Cells were transfected with cDNA encoding Kv1.3 and v-src as described above except that the transfection medium contained 10 μg/mL brefeldin A. The transfection medium

was replaced with normal medium containing 10 μg/mL brefeldin A, and the cells were harvested under standard conditions 36 hours following the initiation of the transfection procedure. Western blot analysis and visualization of protein tyrosine phosphorylation were performed as described above.

Results

The results of this experiment demonstrate that localization of Kv1.3 to the cell membrane is necessary for inhibition of co-expressed v-src. The protein tyrosine phosphorylation levels were analyzed by western blot for four sets of cells: (1) control, (2) expression of v-src only, (3) expression of Kv1.3 only, (4) co-expression of v-src and Kv1.3. Cells in all four runs were treated with brefeldin A, an inhibitor of the Golgi complex that prevents the targeting of newly synthesized proteins to the cell membrane (Klausner *et al.*, (1992), *supra*). The results, displayed in Figure 4, indicated that Kv1.3 expressed alone and prevented from reaching the cell membrane has no effect on protein tyrosine phosphorylation, relative to the control. Expression of v-src alone again resulted in a large increase in protein tyrosine phosphorylation relative to the control. Co-expression of v-src and Kv1.3 localized in the cell interior resulted in a level of protein tyrosine phosphorylation indistinguishable from that observed when v-src is expressed alone. Thus Kv1.3 exhibits inhibition of co-expressed v-src only when located within the cell membrane.

Example 8 Effect of an active Kv1.3 mutant (Y449F Kv1.3) on endogenous PTK activity

Materials and methods

Transfection and growth of cells, pervanadate treatment, cell lysis, western blot analysis and phosphotyrosine visualization were performed as described above.

Preparation of Y449F Kv1.3

Site-directed mutagenesis was used to construct a Kv1.3 mutant (Y449F Kv1.3) in which tyrosine residue 449 is substituted by phenylalanine. The procedure employed was identical to that described in Example 6 for the preparation of W386F Kv1.3 with one exception. In this example the restriction enzyme BbrP1 (Boehringer Mannheim, Indianapolis, IN) was used rather than Pst1 as described in Example 6.

Results

The results of this experiment show that increasing the open time of a potassium ion channel increases its ability to inhibit PTK activity. Recent work has implicated phosphorylation of tyrosine 449 as a major cause of Kv1.3 inactivation. Replacement of this residue with phenylalanine was thus expected to increase the open time of the potassium ion channel in the presence of PTKs. Figure 7 compares the effects of expression of Kv1.3 and Y449F Kv1.3 on endogenous PTK activity, as unmasked by pervanadate treatment, and shows that Y449F Kv1.3 inhibited PTK activity in the cell to a greater extent than Kv1.3. Thus, increasing the open time of the channel, or making the channel more resistant to inactivation, increases its ability to attenuate PTK activity.

Example 9 The effect of valinomycin on cellular PTK activity

Materials and methods

A stock valinomycin solution (5 mg/mL valinomycin in ethanol) was diluted 1:1000 in serum-free MEM.

Treatment of cells with valinomycin

Cells were divided into sets 1-4 for this study. Sets 3 and 4 were transfected with cDNA encoding Kv1.3 as described above. All sets of cells were washed 3 times with serum-free MEM. Sets 1 and 3 were incubated for 10 minutes with serum-free MEM, while sets 2 and 4 were incubated for 10 minutes with serum-free MEM plus 5 μg/mL valinomycin. All groups were then treated for 30 minutes with a solution of 250 μM pervanadate in serum-free MEM, which for sets 2 and 4 also contained 5 μg/mL valinomycin. Cell lysis, western blot analysis and phosphotyrosine visualization were performed as described above.

Results

This experiment shows that valinomycin inhibits endogenous PTK activity as unmasked by pervanadate. Figure 8 shows the results of phosphotyrosine analysis for the four sets of cells (set 3 not shown). Cells from set 2, treated with valinomycin but not expressing Kv1.3, showed a decrease in phosphotyrosine level of approximately 40% relative to set 1. Cells from set 4, which were treated with valinomycin and express Kv1.3, showed a slight drop in tyrosine phosphorylation relative to set 2.

Valinomycin is a potassium-selective ionophore which forms a membrane-permeant complex with potassium ion. These results demonstrate that cellular PTK activity can be inhibited through an increase in cell membrane potassium

ion conductance by a route independent of potassium ion channel proteins.

EQUIVALENTS

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

We claim:

1. A method for treating a tyrosine kinase-mediated condition comprising administering to a mammal an effective amount of an agent that increases cell membrane potassium ion conductance.

2. The method of Claim 1 wherein the condition is cancer.

3. The method of Claim 1 wherein the condition is psoriasis or atherosclerosis.

4. The method of Claim 2 wherein the cancer is selected from the group consisting of leukemia, breast cancer, skin cancer, lymphoma, brain cancer, ovarian cancer, stomach cancer, glioma, bladder cancer, thyroid cancer and prostate cancer.

5. The method of Claim 1 wherein the agent that increases cell membrane potassium ion conductance is a potassium ion channel activator.

6. The method of Claim 5 wherein the potassium ion channel activator is minoxidil.

7. The method of Claim 5 wherein the potassium ion channel activator is a benzimidazolone compound.

8. The method of Claim 1 wherein the agent which increases cell membrane potassium ion conductance is a compound which with a potassium ion forms a

hydrophobic complex which is capable of passage through the cell membrane.

9. The method of Claim 1 wherein the agent that regulates cell membrane potassium conductance is a potassium ion channel protein.

10. The method of Claim 9 wherein the potassium ion channel protein is selected from the group consisting of Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv1.7, Kv2.1, Kv3.1, Kv3.2, Kv3.3, Kv3.4, Kv4.1, and Kv4.2.

11. The method of Claim 9 wherein the potassium ion channel protein is a genetic mutant of a wild-type potassium ion channel protein.

12. The method of Claim 11 wherein the genetic mutation of a wild-type potassium ion channel protein removes one or more phosphorylation sites.

13. The method of Claim 9 wherein the potassium ion channel protein is administered via a vector which, upon introduction into the cell containing the tyrosine kinase, results in expression of an active potassium ion channel.

14. The method of Claim 13 wherein the vector comprises a polynucleotide molecule encoding a potassium ion channel protein.

15. The method of Claim 14 wherein the polynucleotide molecule is a DNA molecule.

16. The method of Claim 15 wherein the vector is selected from the group consisting of adenovirus, cytomegalovirus, herpes simplex virus type 1, and Epstein Barr virus.

17. The method of Claim 14 wherein the polynucleotide molecule is an RNA molecule.

18. The method of Claim 17 wherein the vector is an avian sarcoma virus and or a murine sarcoma virus.

19. The method of Claim 15 wherein the vector is a liposome.

20. A pharmaceutical composition comprising a vector containing a polynucleotide molecule encoding a potassium ion channel protein in a pharmaceutically acceptable carrier.

21. The composition of Claim 20 wherein the polynucleotide molecule is a DNA molecule.

22. The composition of Claim 20 wherein the polynucleotide molecule is an RNA molecule.

23. The composition of Claim 21 wherein the vector is selected from the group consisting of adenovirus, herpes simplex virus type 1, cytomegalovirus and Epstein Barr virus.

24. The composition of Claim 21 wherein the vector is a liposome.

25. The composition of Claim 22 wherein the vector is an avian sarcoma virus or a murine sarcoma virus.

26. The composition of Claim 20 wherein the potassium ion channel protein is selected from the group consisting of Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kv1.7, Kv2.1, Kv3.1, Kv3.2, Kv3.4, Kv3.3, Kv4.1 and Kv4.2.

27. The composition of Claim 20 wherein the potassium ion channel protein is a genetic mutant of a wild-type potassium ion channel protein.

28. The composition of Claim 27 wherein the genetic mutation of a wild-type potassium ion channel protein removes one or more phosphorylation sites.

29. A method for assaying potassium ion channel regulatory activity in cells by a compound, comprising the steps of:

(a) contacting cells with an agent which increases potassium ion channel activity;

(b) contacting the cells of step (a) with an agent which increases tyrosine phosphorylation;

(c) contacting the cells of step (b) with the compound; and

(d) measuring tyrosine phosphorylation.

30. The method of Claim 29 wherein the agent of step (b) is a protein tyrosine phosphatase inhibitor.

31. The method of Claim 30 wherein the protein tyrosine phosphatase inhibitor is pervanadate or orthovanadate.

32. The method of Claim 29 wherein the agent of step (b) is a vector containing a polynucleotide molecule encoding a protein tyrosine kinase.

33. The method of Claim 32 wherein the vector is contacted with the cells under conditions suitable for lipofectamine infection, electroporation, calcium phosphate-mediated incorporation, microinjection, DNA viral infection or retroviral infection.

34. The method of Claim 32 wherein the protein tyrosine kinase is selected from the group consisting of v-src, EGFr and v-abl.

35. The method of Claim 29 wherein the agent of step (a) is a vector containing a polynucleotide molecule encoding a potassium ion channel protein.

36. The method of Claim 35 wherein the polynucleotide molecule is administered to the cells under conditions suitable for lipofectamine infection, electroporation, calcium phosphate-mediated incorporation, microinjection, DNA viral infection or retroviral infection.

37. A method for diagnosing tumor virulence, comprising measuring the cell membrane potassium ion conductance of cancerous cells from a tumor biopsy of a cancer patient.

38. The method of Claim 37 wherein the cell membrane potassium ion conductivity is measured by patch clamp analysis.

39. A mutant of potassium ion channel protein Kv1.3 wherein one or more tyrosine phosphorylation sites are removed.

40. The mutant potassium ion channel protein of Claim 39 wherein the tyrosine residue at position 449 of Kv1.3 is replaced by a phenylalanine residue.

Kv1.3 Expression Decreases V-Src-Induced Protein Tyrosine Phosphorylation

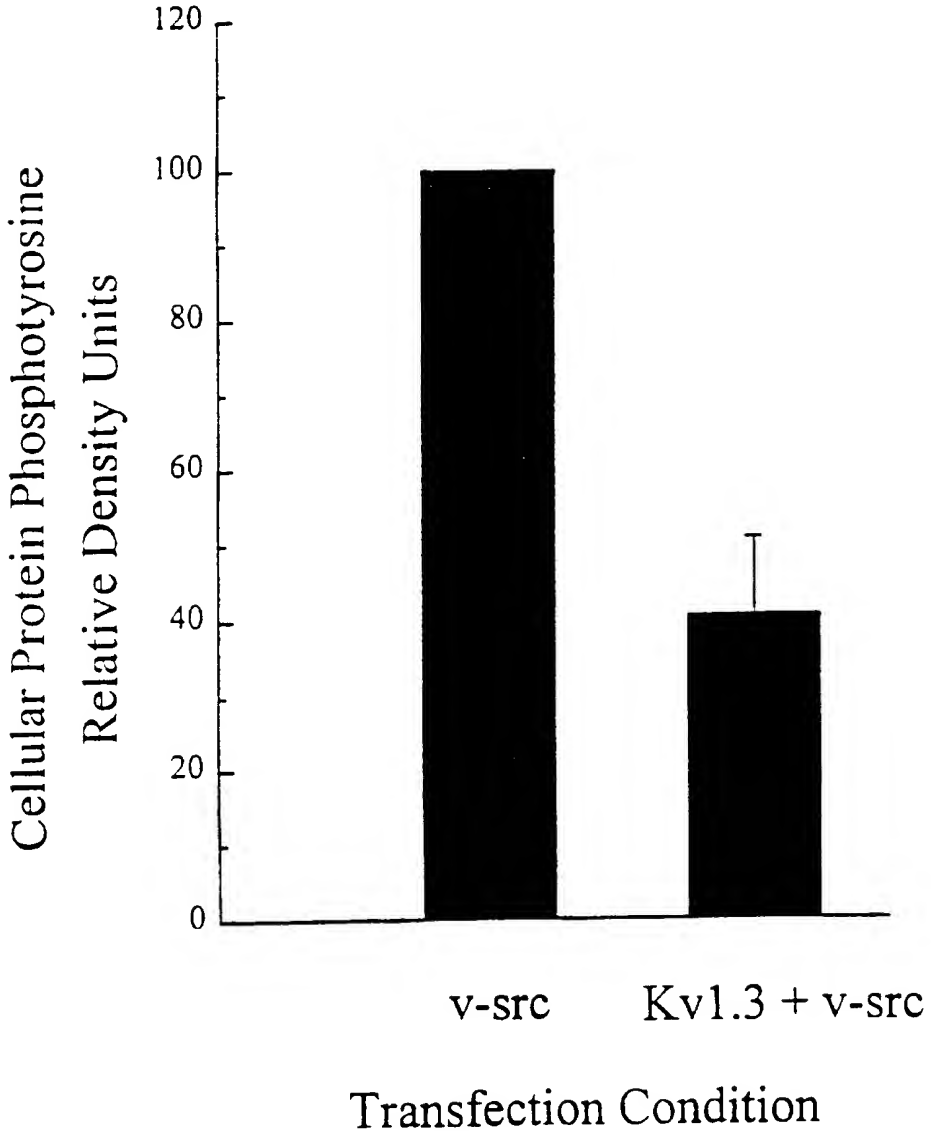

FIGURE 1

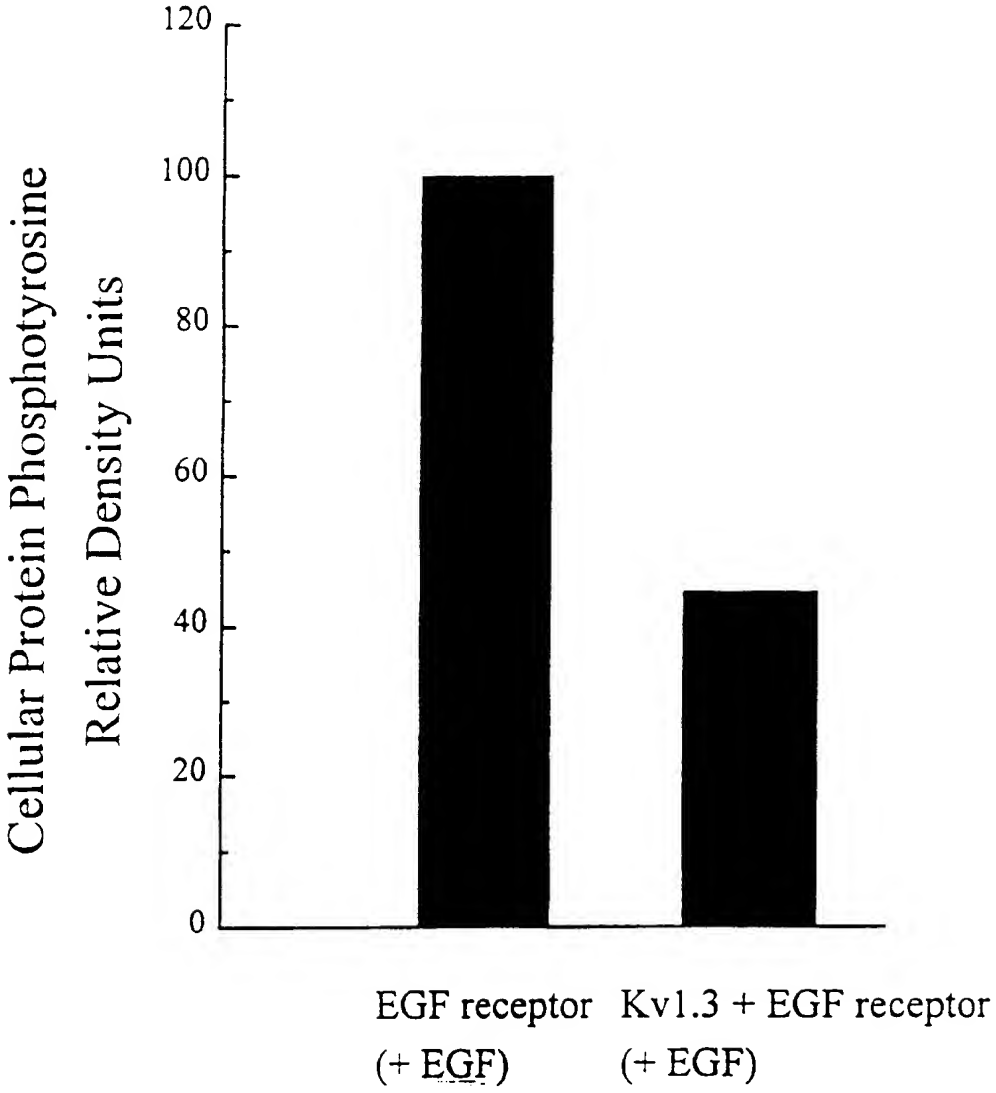
Cellular Protein Phosphotyrosine
Relative Density Units
120
100
80
60
40
20
0
EGF receptor (+ EGF)
Kv1.3 + EGF receptor (+ EGF)

FIGURE 2

Kv1.3 Expression Decreases Pervanadate-Induced Protein Tyrosine Phosphorylation

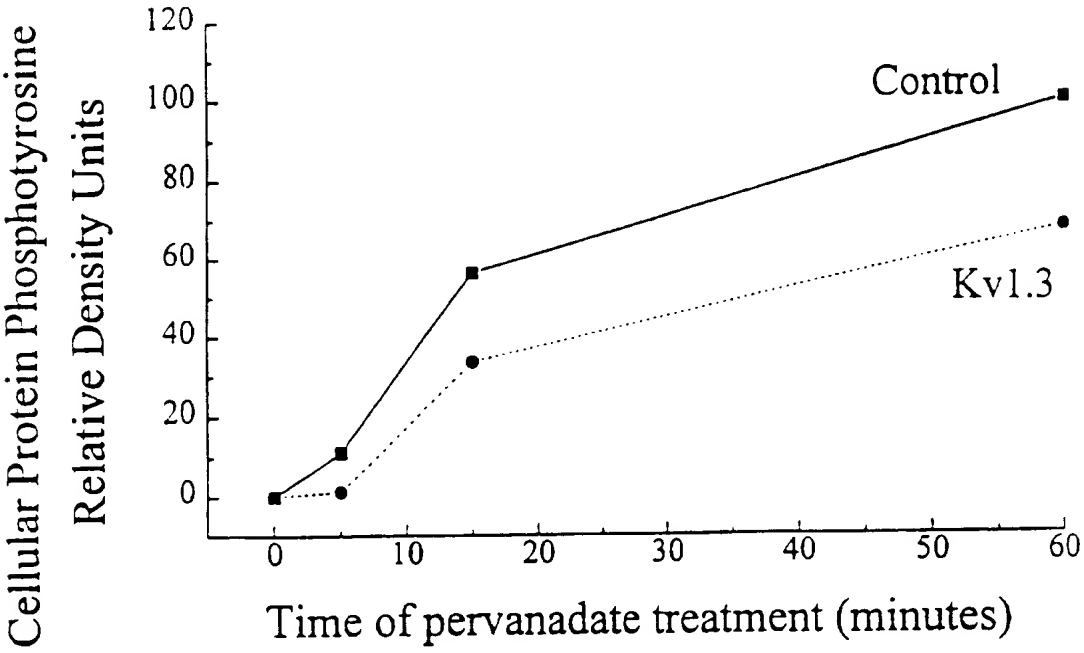

FIGURE 3

Kv1.3 Expression Does Not Affect V-src Protein Expression

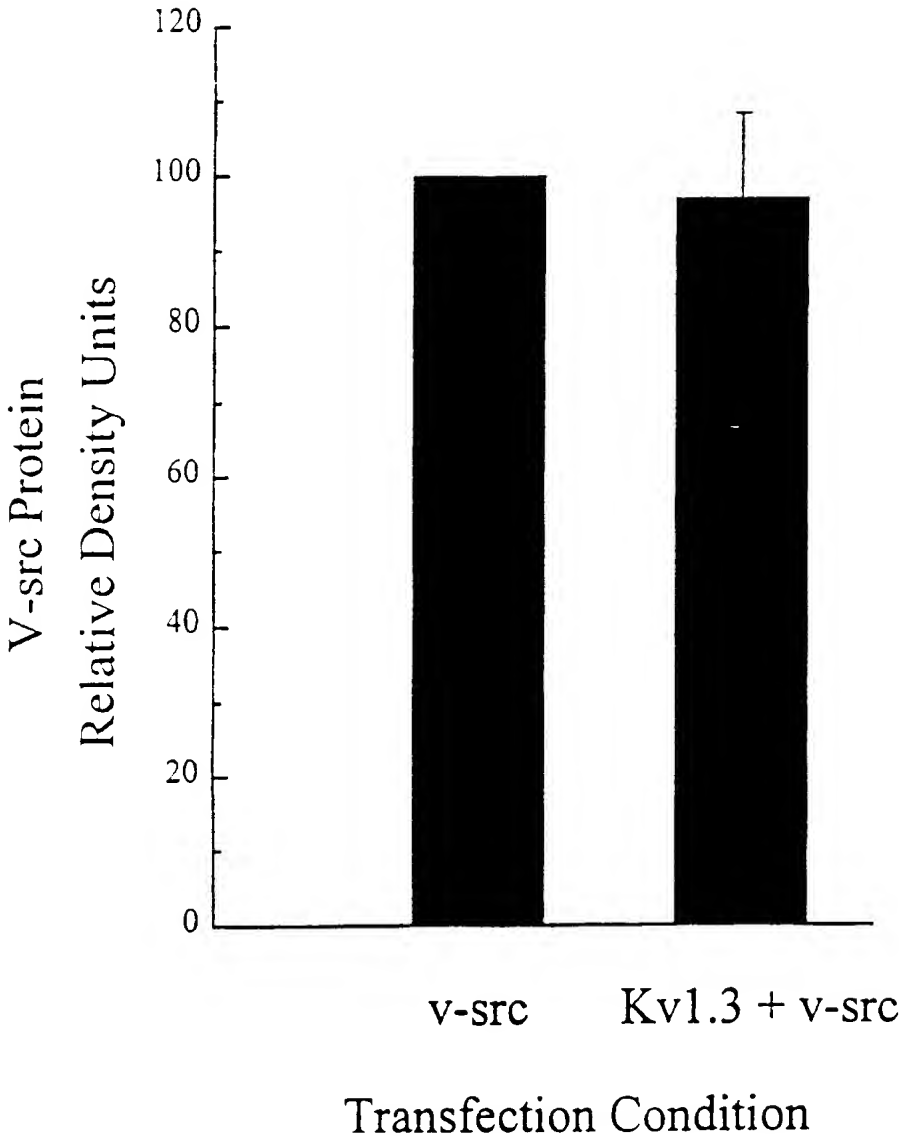

FIGURE 4

Kv1.3 and v-src Expression Does Not Decrease Total Cellular Protein

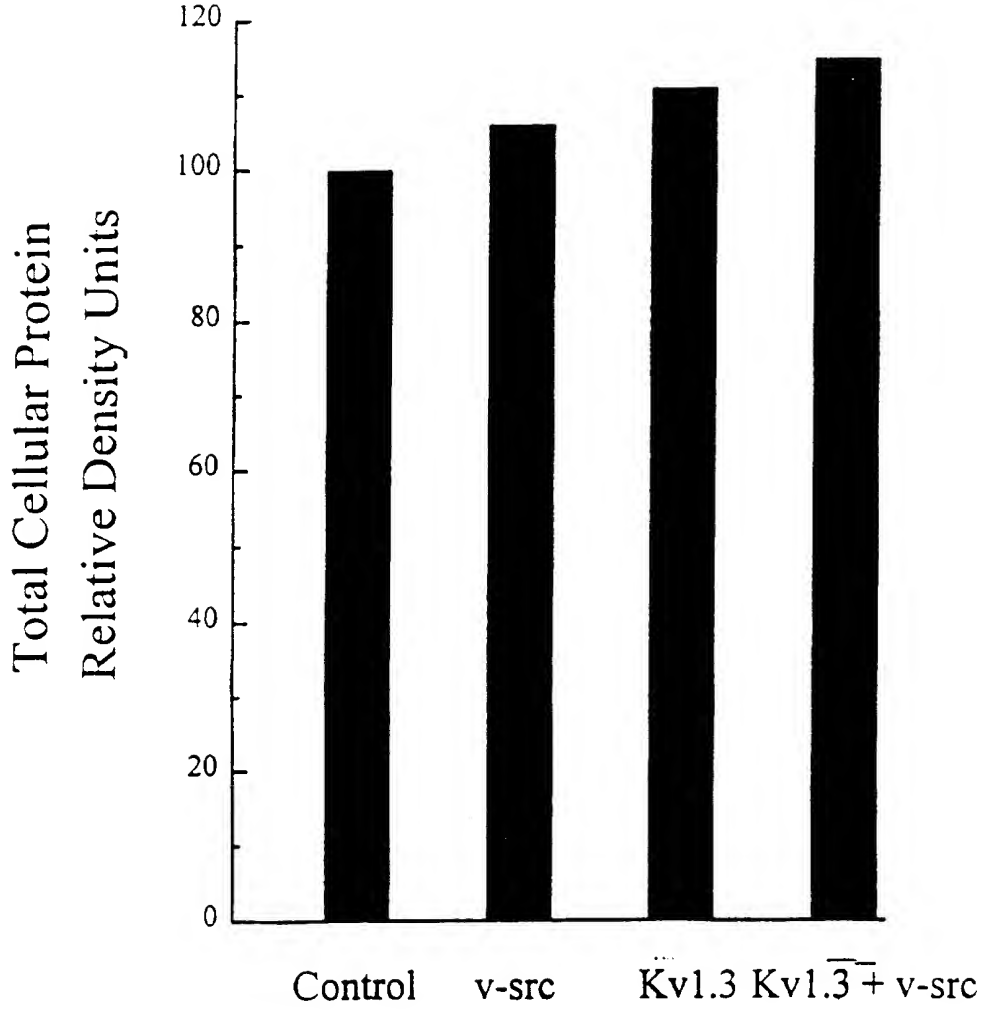

FIGURE 5

Kv1.3 and W386F Kv1.3 Expression and V-src Induced Protein Tyrosine Phosphorylation

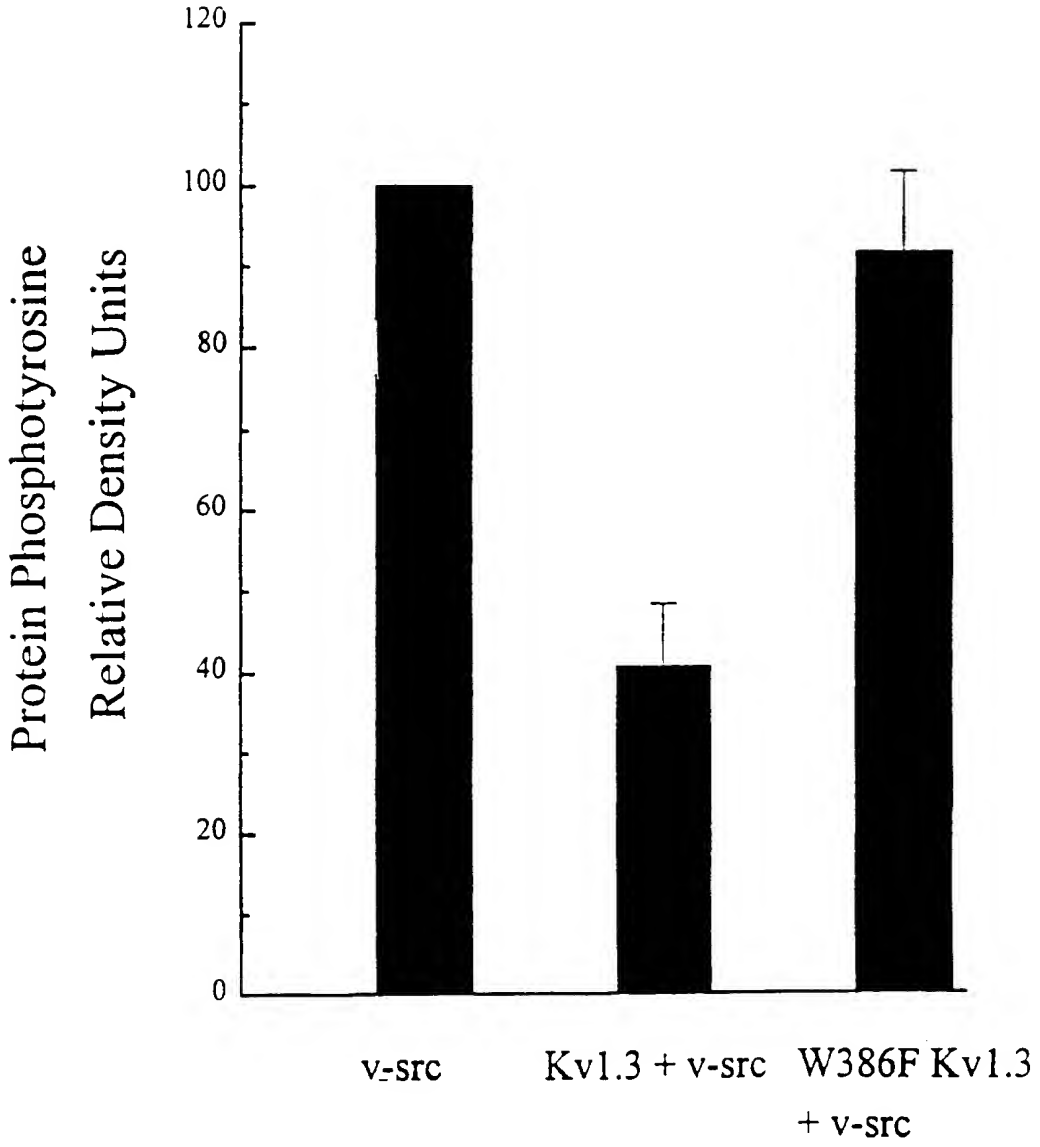

FIGURE 6

Kv1.3 and Y449F Kv1.3 Expression Decreases Pervanadate-Induced Protein Tyrosine Phosphorylation

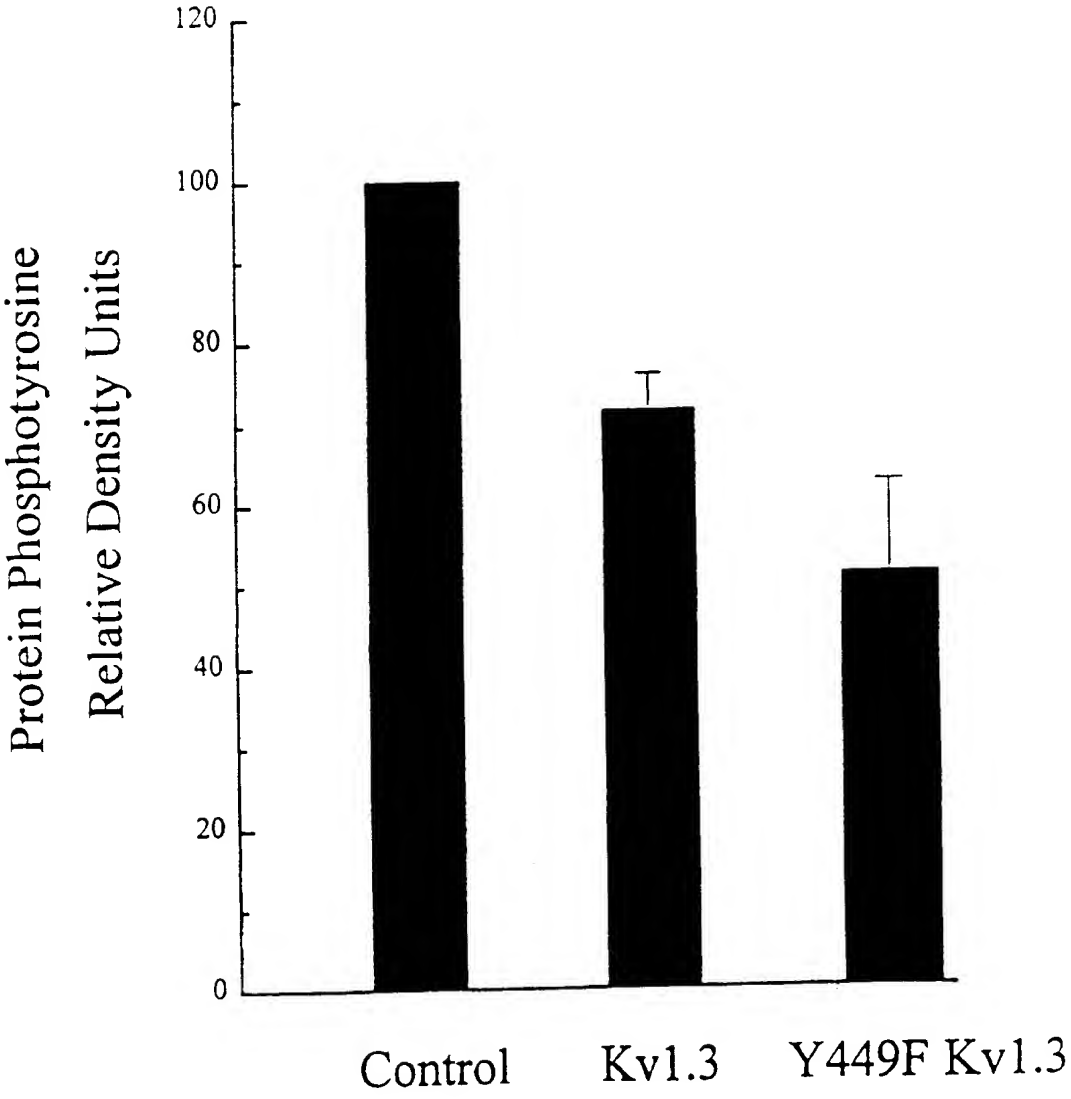

FIGURE 7

Valinomycin Treatment and Kv1.3 Expression with Valinomycin Treatment Decreases Pervanadate-Induced Protein Tyrosine Phosphorylation

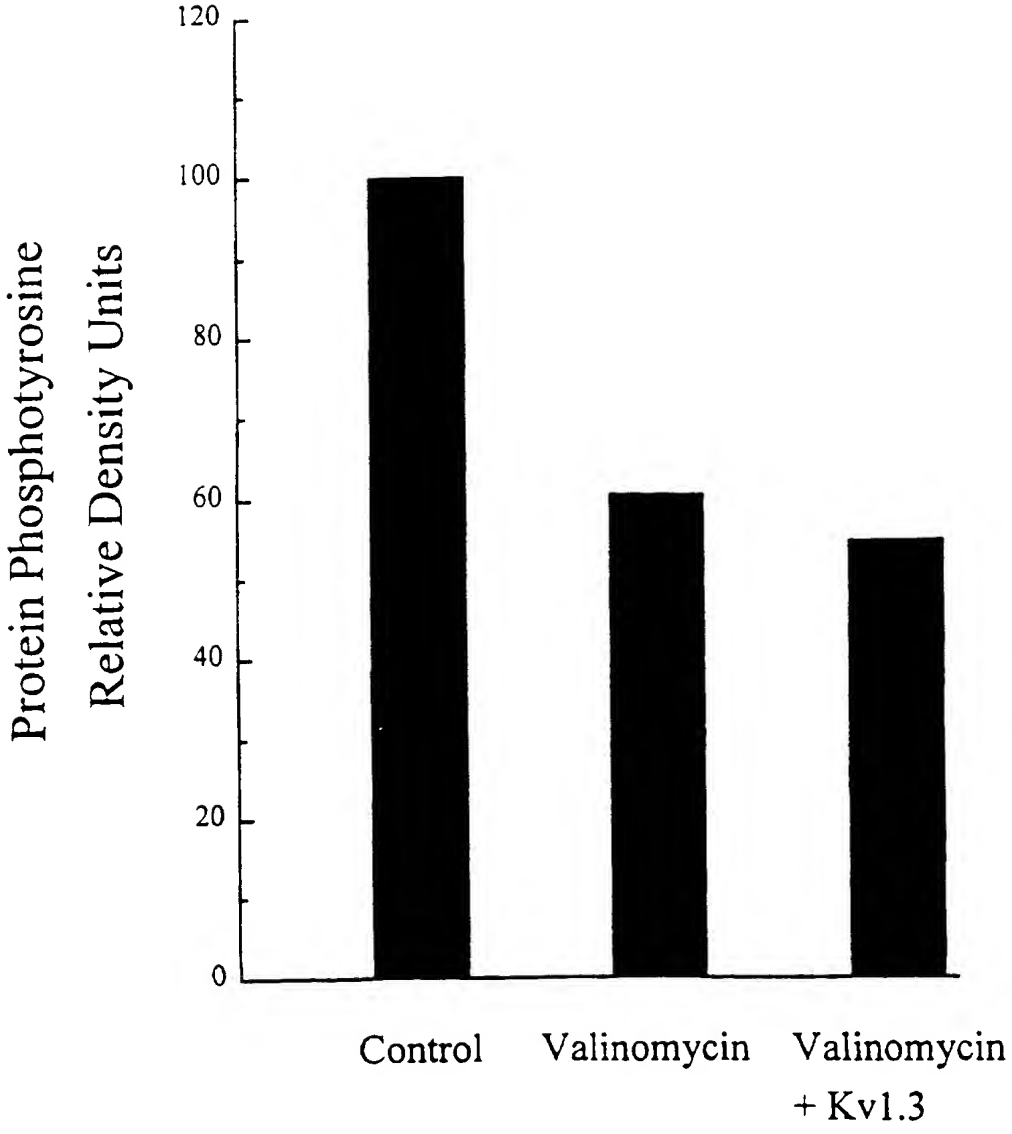

FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18304

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C07K 14/00; A61K 38/18, 38/43

US CL :435/6, 7.6, 9, 14, 16, 21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.6, 9, 14, 16, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SWANSON et al. Cloning and Expression of cDNA and Genomic Clones Encoding Three Delayed Rectifier Potassium Channels in Rat Brain. Neuron. June 1990, Vol. 4, pages 929-939, especially page 930.	9, 10, 20- 28
X --- Y	LEVITZKI et al. Tyrosine Kinase Inhibition: An Approach to Drug Development. Science. 24 March 1995, Vol. 267, pages 1782-1788, see entire document.	1-8, 11-19, 29-40 --- 9, 10, 20- 28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 FEBRUARY 1997

Date of mailing of the international search report

05 MAR 1997

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